# The Effect of Ethanol and Oxygen on the Growth of *Zymomonas mobilis* and the Levels of Hopanoids and Other Membrane Lipids

**Abstract.** *Zymomonas mobilis* (ATCC 29191) was grown either aerobically or anaerobically in the presence of 2% (wt/vol) glucose and 0, 3, or 6% (vol/vol) ethanol. The rates of growth and the composition of hopanoids, cellular fatty acids, and other lipids in the bacterial membranes were quantitatively analyzed. The bacterium grew in the presence of 3% and 6% ethanol and was more ethanol tolerant when grown anaerobically. In the absence of ethanol, hopanoids comprised about 30% (by mass) of the total cellular lipids. Addition of ethanol to the media caused complex changes in the levels of hopanoids and other lipids. However, there was not a significant increase in any of the hopanoid lipid classes as ethanol concentration was increased. As previously reported, vaccenic acid was the most abundant fatty acid in the lipids of *Z. mobilis*, and its high constitutive levels were unaffected by the variations in ethanol and oxygen concentrations. A cyclopropane fatty acid accounted for 2.6–6.4 wt % of the total fatty acids in all treatments.

*Zymomonas mobilis*, a Gram-negative bacterium, has previously been shown to both produce ethanol and tolerate the presence of high levels of ethanol in the medium. Because *Z. mobilis* has been reported to be able to produce ethanol at rates and efficiencies that rival or surpass those of yeasts, there has been considerable interest in the use of this bacterium for the production of fuel ethanol [8, 9]. Two possible mechanisms have been proposed to explain the high degree of ethanol tolerance exhibited by this bacterium. In the first mechanism, it is postulated that the high levels of hopanoids (pentacyclic triterpene lipids) in the cell membranes protect the bacteria from the toxic effects of ethanol [1, 3, 7]. In the second mechanism, it is postulated that the high levels of *cis*-vaccenic acid in the phospholipids of the bacterial membrane protect the bacterium from ethanol toxicity [2]. Although three previous reports [1, 3, 7] have attempted to examine the relationship between the levels of ethanol in the medium and the levels of hopanoids in *Z. mobilis*, the HPLC method used in the current study was more accurate than those employed previously, and it permitted the simultaneous analysis of the levels of three types of hopanoids and four types of phospholipids for each treatment.

## Materials and Methods

**Bacterial strains and culture conditions.** *Z. mobilis* ATCC 29191 and ATCC 31821 were obtained from the American Type Culture Collection (Rockville, MD). The strains were maintained on a solid medium, and starter cultures were prepared as previously described [4]. Final cultures were grown in 2.8-L Fernbach flasks, each containing one liter of fresh liquid medium (2% glucose, 1% yeast extract, 0.2% $KH_2PO_4$, pH 6.0), at 28°C, with shaking (100 rpm). When ethanol was added to the media, it was added as 100% ethanol, pipetted directly into aqueous media that had previously been autoclaved and cooled to room temperature. Cells were harvested by centrifugation (4700 *g*, 30 min), the cell pellet was washed once with distilled water, recentrifuged, lyophilized, weighed, and stored at −20°C (storage time varied from 1 to 30 days with no apparent change in lipid composition). Glucose concentrations were measured with a Yellow Springs Instruments (Yellow Springs, OH) Model 2000 glucose analyzer. For estimating biomass during incubation, the optical density at 600 nm was measured with a Beckman Model 35 dual beam spectrophotometer (Beckman Instruments, Fullerton, CA).

**Lipid extraction and chromatography.** Lyophilized bacterial cells (200 mg) were extracted and the total lipid extract (20–50 µl of 10

mg/ml) was injected directly into the HPLC-flame ionization detector (FID) to quantitatively analyze the levels of intact (nonderivatized) hopanoids, phospholipids, and nonpolar lipids, as previously described [4]. For analysis of fatty acids (derived mainly from phospholipids), an aliquot of total lipid extract that contained 5 mg of lipid was hydrolyzed with methanolic KOH (1.5 N, 1 h, 75°C). The samples were cooled, acidified to a pH of less than 2.0 with 2.0 N HCl, and the hydrolyzed lipids were extracted with chloroform–methanol as previously described [4]. Fatty acid methyl esters (FAMES) were prepared by treating the samples with diazomethane. FAMES were separated and quantified by GLC-FID with a Perkin-Elmer Autosystem GC Plus gas chromatograph and Model 1022 data system (Perkin Elmer, Norwalk, CT). The chromatograph was fitted with an SP 2330 fused-silica capillary column (0.25 mm id × 15 m, Supelco, Bellefonte, PA). The column oven was temperature programmed to increase from 125° to 170°C over 20 min. Injector and detector temperatures were 200 and 250°C respectively, and the carrier gas (He) head pressure was 69 k Pascals. Authentic FAME standards (myristate, myristoleate, palmitate, pamitoleate, and *cis*-vaccenate) were purchased from Sigma Chem. Co. (St. Louis, MO).

Each experiment was repeated at least two times. The results presented are from a single experiment, but are representative of each replicate. All analyses were performed in duplicate, and the values presented are the mean ± standard deviation. Statistical evaluation was performed by application of the Scheffe technique [6]. Means within each atmosphere with no letter subscripts in common are significantly ($p < 0.05$) different.

## Results and Discussion

**The effect of ethanol on the growth of cells of *Z. mobilis*.** ATCC 29191 in the 0% ethanol, aerobic and 0% ethanol, anaerobic culture conditions continued to grow for 43 h, when all of the glucose was depleted from the medium (Fig. 1). At this time the bacteria were harvested and their lipids were extracted. The rate of cell growth was consistently slightly slower in the presence of oxygen than under anaerobic conditions. The addition of ethanol to the media resulted in about a 50% inhibition of cell mass (estimated by $OD_{600nm}$ readings) and a 60–65% reduction in glucose utilization over 43 h in the 3% ethanol, aerobic, or anaerobic, and the 6% ethanol, anaerobic treatments. Cells in the 6% ethanol, aerobic treatment grew extremely slowly and exhibited the slowest rate of glucose utilization.

When compared with *Z. mobilis* ATCC 29191, ATCC 31821 grew more rapidly and was more ethanol tolerant (data not shown). The increased ethanol tolerance of ATCC 31821 compared with ATCC 29191 was also reported by Hermans et al. [3].

**The effect of ethanol on the levels of hopanoids and other lipids in *Z. mobilis*.** Each of the three major hopanoids previously reported in *Z. mobilis* [4] were detected in all cell extracts of ATCC 29191 (Table 1). The chemical structures of these three major hopanoids are shown in Fig. 2A. A sample HPLC-FID chromatogram showing the separation of hopanoids and other lipids in an extract of *Z. mobilis* is shown in Fig. 2B. In the absence of ethanol, hopanoids comprised about 30% (by mass) of the total cellular lipids (Table 1). The addition of ethanol to culture media caused complex changes in the levels of hopanoids and other lipids. When the cells were grown either aerobically or anaerobically, the addition of ethanol (3% or 6%) reduced the levels of total hopanoids. Among the three hopanoids, the levels of THBH were apparently affected by oxygen in the absence of ethanol; the 0% ethanol, anaerobic treatment had the lowest levels of THBH in any of the six treatments. The levels of THBH-GA were reduced 32–71% by the addition of ethanol, under both aerobic and anaerobic conditions. The levels of THBH-ET were reduced significantly (from 78% to 88%) by the 6% ethanol treatment, under anaerobic and aerobic conditions, respectively.

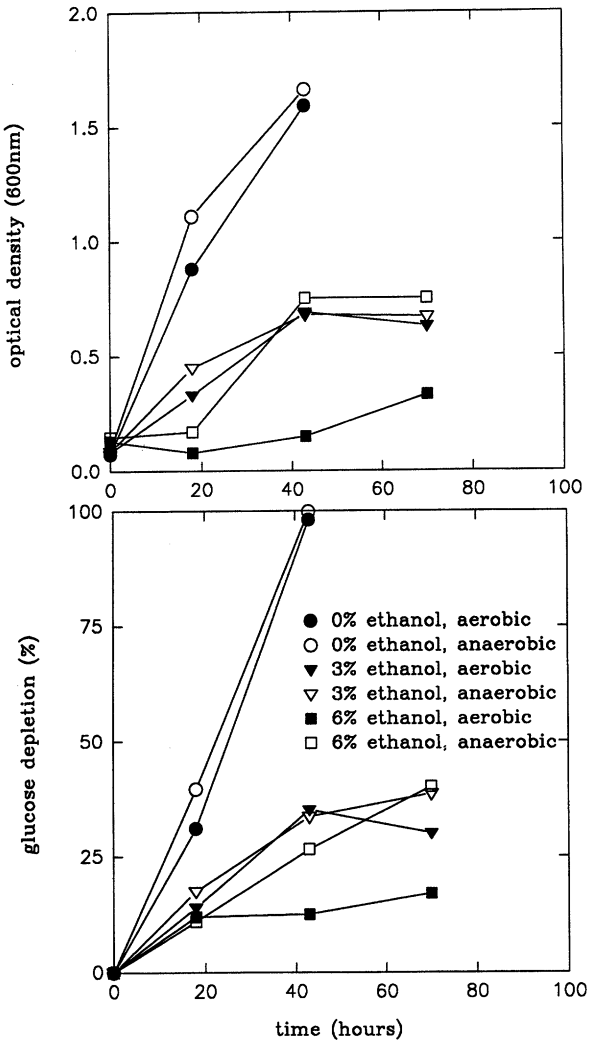



Fig. 1. Effect of ethanol (0, 3, and 6%, vol/vol) on the growth of *Z. mobilis* ATCC 29191, when grown on 2% glucose, under aerobic and anaerobic conditions. (Top) Optical density at 600 nm vs time. (Bottom) Glucose utilization vs time.

Table 1. Quantitative analysis of hopanoids and other lipids in *Zymomonas mobilis* ATCC 29191, when grown aerobically or anaerobically in a medium containing glucose (2%, wt/vol) and ethanol (0, 3, and 6%, vol/vol)

| Lipid class | Aerobic | | | Anaerobic | | |
|---|---|---|---|---|---|---|
| | 0% EtOH | 3% EtOH | 6% EtOH | 0% EtOH | 3% EtOH | 6% EtOH |
| | (mg lipid/gram dry weight of cells ± SD[a]) | | | | | |
| Nonpolar lipids | | | | | | |
| VNPL[b] | 2.91 ± 0.35 c[c] | 4.93 ± 0.16 a | 3.85 ± 0.04 b | 2.63 ± 0.13 b | 4.17 ± 0.40 a | 3.54 ± 0.18 a |
| FFA | 0.48 ± 0.03 b | 0.69 ± 0.02 a | 0.42 ± 0.03 b | 0.40 ± 0.03 b | 0.71 ± 0.00 a | 0.27 ± 0.08 b |
| Hopanoids[b] | | | | | | |
| THBH | 6.53 ± 0.35 a | 5.81 ± 0.18 b | 5.56 ± 0.06 b | 4.83 ± 0.04 b | 7.26 ± 0.49 a | 6.61 ± 0.34 a |
| THBH-GA | 5.89 ± 0.30 a | 3.68 ± 1.12 ab | 2.42 ± 0.41 b | 7.53 ± 0.04 a | 3.93 ± 0.51 b | 2.18 ± 0.32 c |
| THBH-ET | 4.23 ± 0.10 a | 1.15 ± 0.78 b | 0.53 ± 0.04 b | 5.73 ± 0.04 a | 2.06 ± 1.16 b | 1.25 ± 0.78 b |
| Phospholipids[b] | | | | | | |
| CL | 5.74 ± 0.28 a | 7.05 ± 0.89 a | 2.54 ± 0.32 b | 5.83 ± 0.18 a | 6.43 ± 0.67 a | 6.62 ± 0.60 a |
| PE | 15.22 ± 0.28 a | 13.77 ± 0.03 b | 15.67 ± 0.09 a | 16.46 ± 0.08 a | 14.01 ± 0.74 ab | 11.81 ± 1.08 b |
| PG | 0.90 ± 0.03 b | 0.67 ± 0.07 b | 1.56 ± 0.13 a | 1.41 ± 0.16 a | 1.16 ± 0.11 a | 0.30 ± 0.04 b |
| "PI" | 4.70 ± 0.48 a | 5.03 ± 1.40 a | 1.02 ± 0.22 b | 4.12 ± 0.14 a | 4.68 ± 1.36 a | 2.49 ± 0.47 a |
| PC | 6.97 ± 0.10 a | 8.43 ± 0.01 a | 5.49 ± 1.41 a | 6.65 ± 0.10 b | 8.01 ± 0.10 a | 6.55 ± 0.32 b |
| Total hopanoids | 16.65 ± 0.47 a | 10.64 ± 1.38 b | 8.51 ± 0.42 c | 18.09 ± 0.07 a | 13.25 ± 1.36 b | 10.04 ± 0.91 c |
| Total phospholipids | 33.53 ± 0.63 a | 34.95 ± 1.66 a | 26.28 ± 1.47 b | 34.48 ± 0.31 a | 34.29 ± 1.69 a | 27.77 ± 1.36 b |
| Total lipids | 53.57 ± 0.86 a | 51.21 ± 2.16 b | 39.06 ± 1.53 c | 55.60 ± 0.34 a | 52.42 ± 2.21 b | 41.62 ± 1.65 c |
| Hopanoids/phospholipids | 0.497 ± 0.017 a | 0.304 ± 0.042 b | 0.324 ± 0.024 b | 0.525 ± 0.005 a | 0.386 ± 0.044 b | 0.362 ± 0.037 b |

[a] Cell biomass (estimated by determining OD 600 nm) and glucose concentrations in the media were monitored daily and cells were harvested at the last data point recorded for each treatment (see Fig. 1).

[b] For abbreviations see Fig. 2.

[c] Within each atmosphere, means in the same row with no letter in common are significantly different ($p < 0.05$) by Scheffe's test [6].

Among the phospholipids, CL, PE, PG, and PC were quantitatively analyzed. In addition, a lipid that co-chromatographed with PI, and was previously [4] identified as PI, was recently found to be resistant to treatment by base (1.5 N methanolic KOH, 30 min, 70°C), indicating that it does not contain ester bonds. Therefore, this lipid is referred to as "PI" in this report, and we are in the process of characterizing its chemical composition. We have grouped "PI" values with the phospholipids in Table 1, but it may be necessary to revise this grouping when its chemical structure is determined.

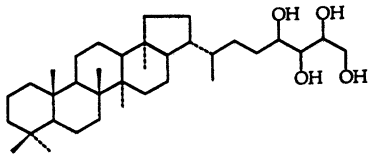
The levels of total phospholipids were unaffected by 3% ethanol, but were decreased 22% and 19% in the two 6% ethanol treatments (aerobic and anaerobic, respectively). Among the individual phospholipid classes, the levels of CL were decreased significantly (approximately 55%) by the 6% ethanol, aerobic treatment. The levels of "PI" were significantly decreased in the 6% ethanol, aerobic treatment. The levels of PE were decreased by both anaerobic, ethanol treatments, but were unaffected by ethanol in the aerobic treatments except with 3% EtOH. Increasing concentrations of ethanol caused some changes in the levels of PG and PC, but no definite trends were observed.

The ratios of total hopanoids/total phospholipids ranged from 0.497 to 0.525 in control (minus ethanol) treatments. The addition of ethanol decreased this ratio significantly under both anaerobic and aerobic conditions.

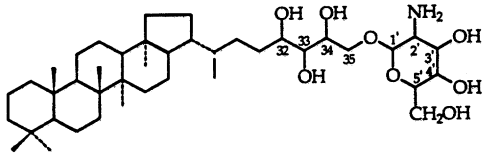
Similar growth studies (± ethanol) and lipid analyses were conducted with a second strain of *Z. mobilis*, ATCC 31821. Comparable results were observed except that, when this strain was grown anaerobically, the addition of 3% or 6% ethanol caused a fourfold increase in the levels of THBH (data not shown).

**The effect of ethanol on the levels of cellular fatty acids in *Z. mobilis*.** Vaccenic acid was the most abundant fatty acid (70–72 wt%) in *Z. mobilis* (Table 2). The levels of vaccenic acid were only slightly affected by oxygen or ethanol, confirming earlier reports [2, 5]. The addition of ethanol significantly increased the levels of palmitic acid. These increases in the levels of palmitic acid were offset by decreases in the levels of three other fatty acids (myristic, myristoleic, and palmitoleic acids.) An unknown fatty acid whose presence (but whose chemical structure was not determined) was previously reported to occur in this species [2] accounted for 2.6 to 6.4 wt% of total fatty acids. Its methyl ester had the longest GLC retention time of all the bacterial FAMES (~11.1 min absolute and 1.17 relative to *cis*-vaccenate), and coeluted with a saturated FAME fraction from a small column of silicic acid impregnated with 10% $AgNO_3$. From this information, the unknown was tentatively identified as

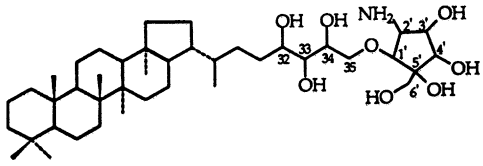
A.



Bacteriohopanetetrol (THBH)



THBH-glucosamine



THBH-ether

B.

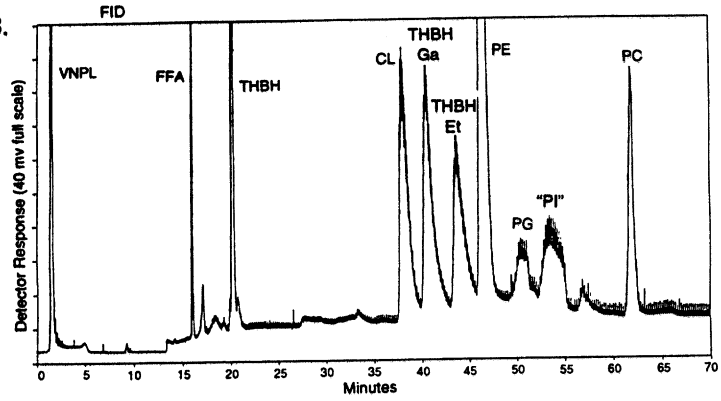



Fig. 2. A. Chemical structures of the three major hopanoids in *Z. mobilis*. B. A representative HPLC chromatogram used for the quantitative analysis of hopanoids and other major lipid classes in *Z. mobilis*. Abbreviations used: VNPL, very nonpolar lipids such as hydrocarbons and triacylglycerols; FFA, free fatty acids; THBH, tetrahydroxybacteriohopanetetrol; THBH-GA, tetrahydroxybacteriohopane-glucosamine, THBH-ET, tetrahydroxybacteriohopane-ether; CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; "PI," an unknown lipid that elutes with the same retention time as phosphatidylinositol (see explanation in Results); PC, phosphatidylcholine.

11,12-methyleneoctadecanoic (lactobacillic) acid, a derivative of *cis*-vaccenic acid found in a variety of bacterial species [2]. This identification was supported by GC-MS analysis, which gave a MW of 310, consistent with the expected formula $C_{20}H_{38}O_2$. The levels of this newly identified fatty acid were not consistently affected by ethanol under aerobic conditions, but were increased 85% and 106% by the presence of 6% ethanol under anaerobic conditions.

In summary, in all treatments there was not a significant increase in any of the hopanoid lipid classes as ethanol concentration was increased. The only exception to this generalization was for THBH, which under anaerobic conditions exhibited a statistically significant increase with both ethanol concentrations. Although we have confirmed that the levels of hopanoids in *Z. mobilis* are not significantly increased (induced) by treatment with ethanol, the question about whether it is the high constitutive levels of hopanoids (or the high constitutive levels of *cis*-vaccenic acid-containing phospholipids) in *Z. mobilis* that permit it to tolerate high levels of ethanol is still open. The recent report of the successful genetic engineering of a strain of *Z. mobilis* to metabolize xylose [9] opens the possibility that *Z. mobilis* could become

Table 2. Analysis of total fatty acids in *Zymomonas mobilis* ATCC 29191, when grown aerobically or anaerobically in a medium containing glucose (2%, wt/vol) and ethanol (0, 3, and 6%, vol/vol)

| Fatty acid | Aerobic | | | Anaerobic | | |
|---|---|---|---|---|---|---|
| | 0% EtOH | 3% EtOH | 6% EtOH | 0% EtOH | 3% EtOH | 6% EtOH |
| | (wt % ± SD[a]) | | | | | |
| Lauric acid (12:0) | 0.4 ± 0.1 a[b] | 0.7 ± 0.1 a | tr b | 0.4 ± 0.3 a | 0.2 ± 0.0 a | tr a |
| Myristic acid (14:0) | 8.8 ± 0.3 a | 7.4 ± 0.4 b | 6.3 ± 0.1 b | 9.0 ± 0.1 a | 7.0 ± 0.0 b | 5.6 ± 0.1 c |
| Myristoleic acid (14:1) | 2.0 ± 0.1 a | 0.6 ± 0.0 b | 0.3 ± 0.0 c | 2.0 ± 0.0 a | 0.7 ± 0.1 b | 0.3 ± 0.0 c |
| Palmitic acid (16:0) | 7.9 ± 0.0 c | 10.5 ± 0.2 b | 15.7 ± 0.0 a | 7.6 ± 0.1 c | 9.6 ± 0.1 b | 12.4 ± 0.1 a |
| Palmitoleic acid (16:1) | 7.3 ± 0.1 a | 6.4 ± 0.1 b | 5.5 ± 0.3 c | 7.5 ± 0.1 a | 6.2 ± 0.1 b | 5.0 ± 0.1 c |
| Stearic acid (18:0) | tr | tr | tr | tr | tr | tr |
| Vaccenic acid (18:1) | 69.8 ± 0.6 a | 69.8 ± 0.4 a | 69.6 ± 0.6 a | 70.5 ± 0.4 b | 71.9 ± 0.4 a | 70.4 ± 0.4 b |
| Lactobacillic acid[c] | 3.8 ± 0.0 b | 4.8 ± 0.2 a | 2.6 ± 0.1 c | 3.1 ± 0 c | 4.5 ± 0.1 b | 6.4 ± 0.2 a |

tr, trace amounts.

[a] Cells were harvested at the last data point recorded for each treatment in Fig. 1.

[b] Within an atmosphere, means in the same row with no letter in common are significantly different ($p < 0.05$) by Scheffe's test [6].

[c] 11,12-methyleneoctadecanoic acid.

widely used by industrial microbiologists. A better understanding of its mechanism of ethanol tolerance may reveal ways to increase its efficiency of ethanol production and lower the cost.

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